Kidney International, Vol. 31 (1987), pp. 1065–1071

LABORATORY INVESTIGATION

The cAMP system in vasopressin–sensitive nephron segments of the vitamin D-treated rat

TOMAS BERL

Department of Medicine, University of Colorado School of Medicine, Denver, Colorado, USA

The cAMP system in vasopressin–sensitive nephron segments of the vitamin D-treated rat. The present study was undertaken to investigate the cAMP system in isolated vasopressin (AVP)-sensitive segments of the hypercalcemic rat. Hypercalcemia was produced by supplementation of diet with dihydrotachysterol, achieving a mean serum calcium of 12.6 mg%. Maximal urinary concentration was only 1982 ± 119 mOsm/kg H_2O in pair, watered hypercalcemic rats when compared to 2478 ± 93 mOsm/kg H_2O in controls ($N = 7$) ($P < 0.01$). Vasopressin stimulated adenylate cyclase activity at concentrations of vasopressin between 10^{-9} and 10^{-7} M was indistinguishable in the outer medullary collecting duct (OMCD) and inner medullary collecting duct (IMCD) of tubules dissected from hypercalcemic rats or normocalcemic rats. Likewise, in situ cAMP accumulation in response to 10^{-7} M AVP was not significantly different in either OMCD or IMCD of hypercalcemic or normocalcemic rats at either isotonic or hypertonic media conditions. In contrast, while 10^{-7} M AVP significantly ($P < 0.05$) increased cAMP accumulation in the medullary ascending limb (MAL) of normocalcemic rats it failed to do so in the MAL of hypercalcemic rats. This failure to accumulate cAMP appears to be due to impairment in AVP-stimulated adenylate cyclase rather than to enhanced phosphodiesterase activity. A similar decrement in glucagon stimulated adenylate cyclase occurred with 10^{-6} M glucagon. The results demonstrate that in chronic hypercalcemia the cAMP system in the OMCT and IMCD of the rat is intact, but the MAL demonstrates abnormal AVP responsiveness due to impaired adenylate cyclase. Such an effect in the MAL may explain the decreased medullary–solute content in hypercalcemia and thereby contribute to the concentrating defect seen in the vitamin D treated rat.

The association of hypercalcemia with polyuria and a renal concentrating defect has been widely recognized in both humans [1, 2] and experimental animals [3, 4]. Recent studies in our laboratory have clearly established the nephrogenic nature of the defect [5]. Furthermore, the concentrating defect and the polyuria are present in animals even when not allowed to become polydipsic [5]. The nature of the renal derangement has also been the subject of intense investigation. On the one hand there is evidence to support the view that a defect in the countercurrent multiplier leads to abnormal concentration of solutes [6, 7], a view supported by the observed decrease in chloride resorption observed in in vivo loop microperfusion experiments [8]. On the other hand, a direct cellular effect of hypercalcemia to decrease the hydroosmotic effect of vasopressin has also been suggested. In this regard, elevation of serosal calcium depresses the hydroosmotic response to vasopressin in the toad bladder [9, 10]; however, no such effect is demonstrable in the cortical collecting duct [11]. Biochemical correlates to these observations have been made in rat medullary slices [12, 13]. It is now well recognized that vasopressin acts to stimulate adenylate cyclase in at least two distinct nephron segments, the collecting duct and the medullary thick ascending limb of Henle's loop [14]. In the former tubular segment the arginine vasopressin (AVP)-dependent cAMP system is involved in the regulation of water and perhaps urea permeability, while in the ascending limb AVP regulates the transport of sodium chloride and thereby the concentration of solutes in the interstitium [15]. Studies employing medullary slices cannot differentiate between these two vasopressin–responsive nephron segments. Experiments conducted in isolated nephron segments of normocalcemic animals have examined only the effects of in vitro acute elevations of calcium in the incubation media [16, 17]. Furthermore, these studies have yielded conflicting results as one in the rat suggests that a defect in the medullary collecting duct [16], while another in the mouse points to the medullary ascending limb as the site of the biochemical defect [17]. In addition, the pathogenesis of the concentrating defect observed in chronic hypercalcemia may not be mimicked by such acute intervention. The present study was designed, therefore, to study the effects of chronic hypercalcemia on cAMP metabolism in tubular segments that are responsive to vasopressin, that is, the outer medullary collecting tubule (OMCT), the inner medullary (papillary) collecting tubules (IMCT) and the medullary thick ascending limb of Henle's loop (MAL). Our results demonstrate an intact cAMP system in the collecting ducts, but impaired generation of the nucleotide in the MAL.

Methods

Studies were performed on male Sprague–Dawley rats (Sasco Inc., Omaha, Nebraska, USA) weighing between 250 and 300 g. The animals were fed a commercially obtained diet (ICN Nutritional Biochemicals, Cleveland, Ohio, USA) or an identical diet to which dihydrotachysterol (Philips Roxan, Columbus, Ohio, USA) 4.25 mg/kg diet was added. We have previously demonstrated that such a maneuver promptly increases serum calcium (by day 3) and remains stable thereafter for another seven days [5]. Since the ad libitum food intake of rats on a vitamin D-supplemented diet is somewhat lower than that of controls, to ensure comparable caloric, protein and mineral intake, food intake of controls was matched to that of hyper-

Received for publication August 13, 1985,
and in revised form March 20 and June 23, 1986

calcemic rats. Likewise, since the water intake of these animals is higher, their water intake was matched to that of control rats. At all times the vitamin D-supplemented hypercalcemic rat and its control were studied together. This was particularly so for the biochemical studies on the microdissected segments in which the dissection and the assay in each pair of rats was performed in the same day so as to minimize the effects of intraassay variability.

Effect of vitamin D supplementation on serum calcium, maximal urinary concentration and tissue solute content

Although we have described the abnormalities in water metabolism that accompany the development of hypercalcemia in a previous study [5], we again determined the maximal urinary concentration in the presence of exogenous vasopressin in seven normocalcemic and seven pair watered rats. Specifically, after six or seven days on the diet, the rats were placed in a metabolic cage, water was withdrawn for 24 hours and the urine was collected over the subsequent six hours. The administration of 500 mU of antidiuretic hormone in oil subcutaneously on the day of dehydration and repeated the next morning when the urine samples were being obtained ensured maximal levels of circulating vasopressin.

Following the concentrating test the animals were anesthetized with phenobarbital (6 mg/100 body wt). Blood for serum calcium was obtained by aortic puncture and the kidneys were removed rapidly and placed in an ice bath for determination of tissue solute employing a modification of the method of Appelbloom et al [18] as previously described from our laboratory [5, 19]. Briefly, the kidneys were cut along their longitudinal axis using a razor blade and scalpel. Sections of the cortex, outer medulla, base and papilla were dissected free, frozen in liquid nitrogen and immediately weighed. Tissues from one kidney were then homogenized for determination of sodium, potassium and urea nitrogen content, while tissues of the contralateral kidney were dried to constant weight (72 hours) for determination of tissue water content. Sodium and potassium were determined by atomic absorption spectrophotometer (Perkins Elmer, Norwalk, Connecticut, USA) while urea nitrogen was measured by autoanlyzer (Technicon Instruments Corp., Tarrytown, New York, USA). The tissue osmolality was measured from the sodium, potassium and urea contents.

Microdissection of tubules

Rats were lightly anesthetized with pentobarbital (3 mg/100 g body wt) and the left kidney was perfused with 10 ml of collagenase medium [137 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 0.33 mM Na_2HPO_4, 1 mM $MgCl_2$, 0.25 mM $CaCl_2$, 10 mM glucose, 10 mM Tris, pH 7.4, 20 U/ml collagenase (Millipore Corp., Freehold, New Jersey, USA), 1 mg/ml hyaluronidase (Sigma, St. Louis, Missouri, USA), and 1 mg/ml bovine serum albumin with 20 U/ml heparin] at an infusion rate of 1 ml/min. The medullary tissue was dissected and sliced to thin strips with direction from cortex to papillary tip. Then the pieces were incubated in an aerated collagenase medium for 60 minutes at 35°C. After incubation the tissues were washed with cold microdissection medium (same as the collagenase medium except 1 mM $CaCl_2$ and no collagenase, hyaluronidase, and bovine serum albumin) and kept in ice while microdissection was performed. Inner medullary collecting ducts (IMCD), outer medullary collecting ducts (OMCD) and thick medullary ascending limb (MAL) were dissected under a stereomicroscope using thin needles. Dissected tubules were transferred to a concave bacteriological slide by aspiration in a small droplet of microdissection medium. The tubule were then photographed to measure the lengths (1 to 2 mm for adenylate cyclase and cAMP phosphodiesterase, 3 to 4 mm for cAMP per sample). The microdissected tubules were studied in the following ways.

Determination of in situ cAMP. The microdissection medium was aspirated off and 2.5 μl of modified Krebs–Ringer buffer (140 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 10 mM glucose, 0.8 mM $CaCl_2$, 10 mM sodium acetate, 2 mM NaH_2PO_4, and 20 mM Tris-HCl, pH 7.4) without (basal) or with 1×10^{-7} M arginine vasopressin (AVP). The samples were covered with vaseline-coated concave slides and were incubated at 30°C for 20 minutes. The incubation was stopped by freezing on dry ice, then the frozen samples were transferred to 12 × 75 glass tubes. A buffer of 100 μl 50 mM sodium acetate, pH 6.2, were added and boiled for three minutes the samples were stored at −20°C until assayed. Since media osmolality can itself affect the generation of cAMP [20], in addition to performing the above studies at a media concentration of 300 mOsm/kg, we also assessed the in situ accumulation of cAMP at a media osmolality that mimics that prevailing in vivo. Thus, the OMCD and MAL were studied also at 800 mOsm/kg and the IMCD at 2000 mOsm/kg H_2O. This was accomplished by adding sodium chloride and urea to the Krebs–Ringer buffer so as to maintain the 2:1 ratio of urea and sodium chloride: for 800 mOsm/kg, 197 mM NaCl and 370 mM urea; for 2000 mOsm/kg, 497 mM NaCl and 980 mM urea.

Following acetylation of the experimental samples and standard, cAMP was determined by radioimmunoassay (RIA kit from New England Nuclear, Boston, Massachusetts, USA). In all cases microdissection and assay of a hypercalcemic and its normocalcemic control were performed simultaneously. The assays were performed in four replications. The quadruplicate determinations were meaned in any given experiment for an "n" of one. The variability of the replicate samples ranged from 3 to 15%.

Adenylate cyclase activity. The effect of hypercalcemia on the activity of adenylate cyclase was studied. Following microdissection of the OMCT, IMCT and MAL described above, the tubules were disrupted (permeabilized) by subjecting them to hypoosmotic shock and freezing. Specifically, after the microdissection fluid was aspirated off, 0.5 μl hypoosmotic medium (1 mM $MgCl_2$, 0.25 mM EDTA, 0.1% bovine serum albumin, and 1 mM Tris, pH 7.4) was added under the microscope; the samples were then immediately frozen by placing them on a block of dry ice. The frozen tubules were thawed once and refrozen, then kept at −80°C until assayed. The basal and hormone stimulated activities of adenylate cyclase were stable at −80°C for at least five days.

The activity of adenylate cyclase was measured according to a modification [21] of the method of Morel, Chabardes and Imbert–Teboul [22]. Slides with original tubular length of 1 to 2 mm were incubated at 37°C for 30 minutes in a final volume of 5.5 μl containing 0.25 mM ^{32}P-ATP (4 to 5 × 10^6 cpm/sample), 1 mM cAMP, 3.8 mM $MgCl_2$, 0.25 mM EDTA, 20 mM creatine phosphate, 1 mg/ml creatine phosphokinase, and 100 mM Tris,

Table 1. Tissue solute content (mOsm/kg H_2O) in the kidneys of normocalcemic (N = 7) and hypercalcemic rats (N = 7)

	Normocalcemic		Hypercalcemic
Cortex			
Non-urea solutes	370 ± 11		361 ± 14
P value		NS	
Total solutes	401 ± 7		396 ± 12
P value		NS	
Outer medulla			
Non-urea solutes	502 ± 29		391 ± 23
P value		<0.025	
Total solutes	623 ± 39		488 ± 47
P value		<0.05	
Inner medulla			
Non-urea solutes	618 ± 58		421 ± 46
P value		<0.025	
Total solutes	1119 ± 79		887 ± 70
P value		<0.05	

pH 7.4, with or without addition of AVP (10^{-7} to 10^{-9} M) or glucagon (10^{-6} M). The medium osmolality was increased to 800 mOsm/kg H_2O by addition of NaCl and urea. The reaction mixture was sealed between two vaseline–coated concave slides and immersed in a water bath. The reaction was stopped by addition of cold 150 μl stopping solution (3.3 mM ATP, 5 mM cAMP, 50 mM Tris HCl, pH 7.6, and ^{3}H-cAMP containing 1×10^4 cpm/sample to determine recovery). Produced ^{32}P-cAMP was separated according to the method of Salomon [23] using Dowex-50-X4 (BioRad, Richmond, California, USA) and aluminum oxide (Woelm Pharma, Germany) columns. Assays on normocalcemic and hypercalcemic rats were analyzed together, and four to six samples were analyzed for each point of adenylate cyclase activity. The variation between replication ranged between 1.3 and 15.6%. Enzyme activity was expressed as fmoles cAMP produced/mm/30 min. The four to six determinations in any given experiment were meaned for an "n" of one.

cAMP phosphodiesterase activity. The effect of hypercalcemia on the activity of cAMP phosphodiesterase was studied. Following microdissection the fluid was aspirated off, 0.5 μl hypoosmotic medium (1 mM $MgCl_2$, 0.25 mM EDTA, 0.1% bovine serum albumin, and 1 mM Tris, pH 7.4) was added under the microscope; the samples were then immediately frozen by placing them on a block of dry ice. The frozen tubules were thawed once and refrozen, then kept at −80°C until assayed. The activity of phosphodiesterase has been found to remain stable at −80°C for at least five days. cAMP phosphodiesterase was assayed in a two step reaction [21]. The first reaction was performed in a buffer containing 10 mM $MgSO_4$, 0.1 mM EDTA, 50 mM Tris, pH 8.0, and 1×10^{-6} M ^{3}H-cAMP (4×10^5 cpm/5 μl, New England Nuclear) as a substrate. The reaction was carried out for 20 minutes at 37°C and the second reaction was performed with 1 mg/ml 5′-nucleotidase [snake venom (naja naja), Calbiochem–Behring Corp., La jolla, California, USA] for 10 minutes at 37°C. The produced ^{3}H-adenosine was separated chromatographically using QAE-Sephadex A-25 (Pharmacia, Piscataway, New Jersey, USA) and counted. All of the assays were performed in four to six replicate. The variation between replication was less than 5%. The results of the four to six replicates were meaned for an "n" of one.

Table 2. Protein content in dissected segments of the outer medullary collecting ducts (OMCD), inner medullary collecting ducts (IMCT) and medullary thick ascending limb (MAL) of normocalcemic and hypercalcemic rats

	Normocalcemic μg/mm		Hypercalcemic μg/mm
OMCD (N = 4)	0.111 ± 0.013		0.103 ± 0.0092
P value		NS	
IMCD (N = 4)	0.108 ± 0.010		0.116 ± 0.012
P value		NS	
MAL (N = 5)	0.097 ± 0.013		0.099 ± 0.0095
P value		NS	

N reflects the number of paired rats in each group. Six to eight tubules were studied in each rat.

Determination of protein. In order to ascertain whether hypercalcemia causes an alteration in the protein content/mm tubule, the protein content of microdissected, measured tubules was determined by the method of Lowry et al [24] after 0.5 μl of 0.1% SDS was added to the tubules.

Comparisons between normocalcemic and hypercalcemic rats were performed by the unpaired Student's t-test. A P value of less then 0.05 was considered significant. The data is presented as the ± standard error of the mean. This SEM was obtained from the mean of number of animals and not all the individual samples.

Results

The seven rats placed on a vitamin D supplemented diet had a mean serum calcium of 12.6 ± 0.4 mg% after six to seven days on the diet as compared to 9.8 ± 0.3 mg% of their seven pair fed and pair watered controls ($P < 0.001$). The maximal urinary osmolality was 2478 ± 93 mOsm/kg H_2O in controls and 1982 ± 119 mOsm/kg H_2O in the hypercalcemic rats ($P < 0.01$). Table 1 summarizes the effect of the hypercalcemia on tissue content. The percent water content was not different in the kidneys of normocalcemic and hypercalcemic rats. The cortical content of solute was not different in the two groups of animals. However, the total tissue solute content was lower both in the outer medulla, and as we noted previously [5], in the inner medulla as well ($P < 0.05$). It is of note that this difference in solute content was entirely accounted for by non-urea solutes (2 + [Na + K]) as the content of urea was comparable in the two groups of animals. More specificially, the increment was in Na rather than K.

In vitro studies

The gross appearance of the tubules obtained from normocalcemic and hypercalcemic rats was not different. As is noted in Table 2, the protein/mm length was not significantly different between normocalcemic and hypercalcemic rats in any of the three nephron segments under study. The data are presented herein relative to tubular length.

Studies in the medullary collecting ducts. Our initial studies were directed to assess whether the OMCD of hypercalcemic rats has an impairment in adenylate cyclase response to vasopressin. As is depicted in the Figure 1A, in five pairs of normocalcemic and hypercalcemic rats the activity of the enzyme obtained from the two groups of animals was essen-

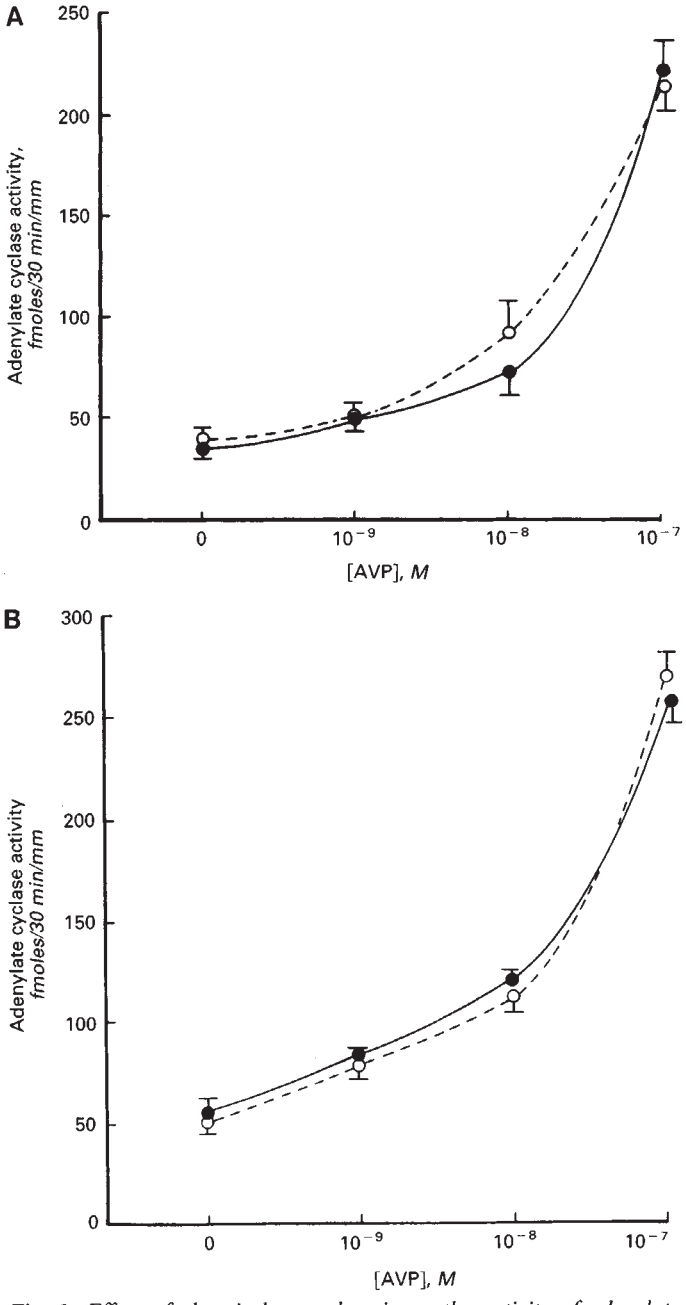

Fig. 1. *Effect of chronic hypercalcemia on the activity of adenylate cyclase at 800 mOsm/kg H_2O in outer medullary collecting ducts (OMCD)* (**A**) *and inner medullary collecting ducts (IMCD)* (**B**). At no concentration of vasopressin was the activity different in the two groups of rats.

tially indistinguishable at concentrations of vasopressin of 10^{-9}, 10^{-8} and 10^{-7} M. Since we recently noted divergent responses to vasopressin in OMCD and IMCD of hypokalemic rats [19] we determined the activity of adenylate cyclase in dissected IMCD. As noted in Figure 1B, again the response in six pairs of normocalcemic and hypercalcemic rats was indistinguishable. Likewise the activity of cAMP-phosphodiesterase studied at a substrate concentration of 10^{-6} M was not found to be different. Specifically, the cAMP-phosphodiesterase activity in OMCD

Table 3. Accumulation of cAMP in the outer medullary collecting ducts (OMCD) and inner medullary collecting ducts (IMCD) of normocalcemic and hypercalcemic rats

	Basal	10^{-7} M AVP
OMCD *fmoles/mm*		
Media osmolality 300 (N = 5)		
Normocalcemic	7.43 ± 2.8	22.1 ± 1.4
Hypercalcemia	5.32 ± 1.5	29.1 ± 3.6
P value	NS	NS
Media osmolality 800 (N = 6)		
Normocalcemic	10.3 ± 2.6	35.1 ± 5.7
Hypercalcemia	8.57 ± 1.1	29.1 ± 6.3
P value	NS	NS
IMCD *fmoles/mm*		
Media osmolality 300 (N = 5)		
Normocalcemic	8.02 ± 3.0	32.1 ± 3.6
Hypercalcemia	8.61 ± 1.2	26.2 ± 4.0
P value	NS	NS
Media osmolality 2000 (N = 7)		
Normocalcemic	8.57 ± 1.1	138.0 ± 10.1
Hypercalcemia	9.75 ± 3.1	165.0 ± 15.7
P value	NS	NS

N denotes the number of paired experiments in each group. Four to six tubules were analyzed in each determination.

was 16.2 ± 1.3 fm/min/mm in normocalcemic rats and 14.9 ± 0.9 fm/min/mm in hypercalcemic rats (N = 4). Likewise, in the IMCD the activities were 11.6 ± 0.89 and 12.3 ± 1.1 fm/min/mm in normocalcemic and hypercalcemic rats, respectively (N = 5).

While the above results would suggest an intact ability to accumulate cAMP in the collecting ducts of hypercalcemic rats, the results obtained with these assays in permeabilized tissues do not always correlate with in situ cAMP accumulation [20]. We therefore measured cAMP in intact tubule in response to vasopressin at 300 and 800 mOsm/kg H_2O in the OMCD and at 300 and 2000 mOsm/kg H_2O in IMCD. The results of those experiments are summarized in Table 3. Neither the basal nor the AVP stimulated accumulation of cAMP was significantly different when tissue from normocalcemic and hypercalcemic were compared in either of these two AVP-responsive nephron segments in isotonic and hypertonic media conditions.

Studies in medullary thick ascending limb. The response of in situ intact MAL to 10^{-7} M AVP in normocalcemic and hypercalcemic rats is shown in Table 4. Despite the fact that the basal activities in the tissue of hypercalcemic rats was somewhat higher, the tissue seemed to be essentially unresponsive to AVP as the measured cAMP was barely, and not significantly higher after the hormones. In contrast, the tissues of the normocalcemic rats sustained a small but consistent increase in cAMP, an increase that was significantly ($P < 0.01$) greater than that of experimental rats.

To assess whether the failure to accumulate cAMP in the MAL of hypercalcemic rats is due to a defect in the generation of the nucleotide, we measured the adenylate cyclase in these tubules from six pairs of rats. Since we find consistent responses only at 10^{-7} M AVP, this concentration of hormone was employed. Table 5 shows the activities in the six experiments. Both normocalcemic and hypercalcemic rats had significant increases in adenylate cyclase activity in response to AVP. However, the increase in hypercalcemic rats was modest and significantly ($P < 0.02$) lower than that of normocalcemic

Table 4. Accumulation of cAMP in medullary ascending limb (MAL) of normocalcemic and hypercalcemic rats in response to 10^{-7} M AVP

	Basal	10^{-7} M AVP
cAMP *fmoles/mm*		
Media osmolality 300 (N = 5)		
Normocalcemic	2.1 ± 0.3	12.1 ± 1.7
Hypercalcemia	3.5 ± 1.1	6.4 ± 1.4
P value	NS	<0.05
Media osmolality 800 (N = 4)		
Normocalcemic	1.6 ± 0.3	16.8 ± 2.3
Hypercalcemia	2.6 ± 1.0	4.0 ± 2.1
P value	NS	<0.01

N denotes the number of paired rats studied. In each case four to six tubules were measured.

controls. To assess whether an increase in cAMP-phosphodiesterase activity could also contribute to the unpaired cAMP accumulation in the MAL we measured the activity of the enzyme in four pairs of rats. This was comparable in the tissue of the two groups (12.31 ± 3.8 in normocalcemic vs. 10.6 ± 3.1 fmoles/min/mm in hypercalcemic).

In order to ascertain whether the impairment in MAL adenylate cyclase is specific to AVP, the response of this segment to glucagon (10^{-6} M) was assessed. Adenylate cyclase increased from 15.48 ± 3.1 to 229.2 ± 26.6 fm/mm/30 min in normocalcemic rats (N = 6), but only from 16.22 ± 2.99 to 144.2 ± 22.8 fm/mm/30 min in the MAL of hypercalcemic rats (N = 6). The response was significantly attenuated in the hypercalcemic rats ($P < 0.05$).

Discussion

A disturbance in renal concentration in hypercalcemia is well recognized. In the present study we undertook to examine the cAMP system in isolated nephron segments of the chronically hypercalcemic rat. Although the biochemical response of renal tissue has been previously measured [12, 13], these determinations in whole medullary tissue homogenate included cells from various segments with different vasopressin responsiveness [14]. More recent studies in which the calcium ion concentrations of the media was acutely changed in isolated tubular segments have yielded conflicting results [16, 17]. Some of the conflict may relate to the difference in species used as well as to the absence or presence of phosphodiesterase inhibitors. In the present study we systematically analyzed the various components of the cAMP system in animals who had a well-established concentrating defect. The degree of the concentrating defect in these rats is not as marked as that encountered when they are allowed ad libitum water intake [5]. However, we chose to pair water in order to prevent the marked polyuria and polydipsia that accompanies hypercalcemia, because these derangements in water turnover can themselves affect the adenylate cyclase system [25, 26]. The pair watering maneuver thus allowed to examine the effects of hypercalcemia without the other confounding variables. Our initial observations were directed to the medullary collecting duct, a nephron segment where vasopressin profoundly affects water permeability. In the chronically hypercalcemic rat neither cAMP accumulation nor adenylate cyclase activity was affected. This was the case both at isotonic and hypertonic conditions. Since we have found that at least in hypokalemia the OMCD and IMCD are not equally afflicted, we assessed the cAMP in IMCD. Unlike the observations we recently made in the hypokalemic rat [19], we found no defect in this nephron segment of the hypercalcemic rat. As was the case in the OMCD, neither cAMP accumulation nor adenylate cyclase activity was affected at either isotonic or hypertonic media conditions. In summary, therefore, our results provide no evidence for a derangement in cAMP metabolism in the collecting ducts of these animals with a well-defined nephrogenic concentrating defect.

Hypercalcemia has been noted to be associated with a decrease in tissue solute [6], an observation we substantiated to be applicable to the inner medulla [5] and in this study the outer medulla as well. It is of note that this decrement in solutes is primarily due to non-urea solutes (primarily sodium) rather than to urea. Since vasopressin appears to be involved in the resorption of solutes in vasopressin as the MAL [27], the next experiments were directed to this segment of the nephron. The accumulation of cAMP both at 300 and 800 mOsm/kg H_2O was impaired in this segment in response to 10^{-7} M AVP. An analysis of the enzymes involved in the generation and breakdown of the nucleotide revealed a decrement in adenylate cyclase but not a change in cAMP-phosphodiesterase. It would appear therefore that the nucleotide generation is impaired in hypercalcemia. Since AVP levels are somewhat higher (12.2 ± 3.6 pg/ml, N = 6) in hypercalcemic rats than in normocalcemic controls (8.8 ± 1.6 pg/ml, N = 6), the possibility that the response is decreased because levels of vasopressin are higher in hypercalcemia, thereby causing a down-regulation of the receptor should be considered. This possibility, however, appears remote since it would not be expected to be limited to only one of the three nephron segments under investigation. In order to ascertain whether the observed impairment in AVP responsiveness is specific to this hormone we tested the effects of 10^{-6} M glucagon. The response to this hormone was also impaired reflecting, not surprisingly, a more generalized ascending limb dysfunction. Since other hormones that act on the ascending limb can also influence urinary concentrating ability the defective response to glucagon may contribute to the defect as well [28].

The relevance of our observations made in vitamin D-treated rats to the concentrating defect in hypercalcemia in general and in man in particular is deserving of comment. Our results reveal that the collecting duct of the hypercalcemic rat has no abnormality in cAMP metabolism. This is in concert with our studies in cultured, renal collecting-duct cells which also failed to show an impairment in cAMP accumulation at extracellular calcium concentrations as high as 4 mM unless cellular calcium also increased [29]. This may well explain the interesting observations of Marx et al who noted that patients with hypercalcemia due to familial hypocalciuric hypercalcemia do not have a concentrating defect while those with primary hyperparathyroidism do [30]. Only in the presence of a calcium ionophore (in this case parathyroid hormone) is the system deranged. The ionophoric effect of PTH in the kidney has not been fully documented. However, the hormone increases cytosolic calcium in proximal tubular cells [31] and enhances ^{45}Ca uptake in a vasopressin sensitive epithelium [32]. Since the vitamin D-treated rat most likely has suppressed levels of PTH, this

Table 5. Activity of adenylate cyclase in MAL of hypercalcemic and normocalcemic rats (fm/mm/30 min)

	Hypercalcemic		Normocalcemic	
Experiment	Basal	10^{-7} M AVP	Basal	10^{-7} M AVP
1	16.6 ± 1.1(5)[a]	38.6 ± 2.3(4)	19.3 ± 1.6(4)	54.4 ± 4.2(4)
2	18.8 ± 0.9(4)	19.5 ± 1.6(5)	15.6 ± 1.9(5)	38.2 ± 3.1(4)
3	2.5 ± 0.2(5)	9.6 ± 1.1(6)	5.4 ± 0.6(5)	25.1 ± 1.6(6)
4	7.2 ± 0.6(4)	14.3 ± 1.3(5)	10.0 ± 1.3(6)	34.6 ± 2.0(4)
5	10.9 ± 1.4(6)	28.8 ± 1.2(5)	19.1 ± 1.8(4)	54.2 ± 3.9(5)
6	16.3 ± 2.0(4)	20.3 ± 1.8(4)	8.6 ± 1.4(5)	41.4 ± 3.2(5)
Mean	12.05	21.85	13.00	41.32[c]
SEM[b]	2.58	4.25	2.38	4.67
P value	<0.05		<0.001	

[a] Denotes ± SEM number of samples is noted in parenthesis
[b] SEM obtained from the number of experiments, not the number of samples
[c] $P < 0.02$ when compared to activity of MAL from hypercalcemic rats

may well explain the failure to observe a biochemical effect on the cyclase system in the model.

The defect in ascending limb solute–reabsorption described in hypercalcemia [8] coupled with the biochemical defect noted in the present studies may well explain the decrement in non-urea solutes and particularly sodium in the medulla of these animals. This decrement in tissue solutes could in itself explain the observed concentrating defect. However, it is not clear whether these mechanisms can be extrapolated to other species in which hypercalcemia causes a concentrating defect, such as the dog and man. Ruggles et al [33] have found no effect of AVP to increase adenylate cyclase in dog or in human kidneys [33, 34]. The possibility that the decrement in glucagon mediated cyclase could alter urinary concentration in this species needs to be considered [28], as deRouffignac et al have demonstrated in hormone deficient models that hormones other than vasopressin contribute to urinary concentration. It must be acknowledged that our studies by no means exclude the possibility that hypercalcemia could exert an effect on the collecting duct whereby its water permeability response to AVP is decreased by altering a biochemical step distal to or entirely independent of cAMP formation. One would have to postulate that such an effect may be operant in species in which the medullary ascending limb is not vasopressin sensitive. The nature of these cAMP effects of hypercalcemia in the collecting duct should be the focus of future investigators.

Acknowledgments

This work was supported by grants from NIH AM 26469 and AM36071. We thank Jin K. Kim, Ph.D. for advice regarding the biochemical assays. I also thank Marilyn Ellis, Ken Vanderslice and Donna Shannon for technical assistance; and Linda M. Benson for secretarial assistance.

Reprint requests to Tomas Berl, M.D., C281, University of Colorado School of Medicine, 4200 E. 9th Avenue, Denver, Colorado 80262, USA.

References

1. Cohen SI, Fitzgerald MG, Fourman P, Griffiths WJ, DeWardener HE: Polyuria in hyperparathyroidism. *Q J Med* 26:423–431, 1957
2. Gill JR Jr, Bartter FC: On the impairment of renal concentrating ability in prolonged hypercalcemia and hypercalciuria in man. *J Clin Invest* 40:716–722, 1961
3. Epstein FH, Rivera MJ, Carone FA: The effect of hypercalcemia induced by calciferol upon renal concentrating ability. *J Clin Invest* 37:1702–1709, 1958
4. Epstein FH, Beck D, Carone FA, Levitin H, Manitius A: Changes in renal concentrating ability produced by parathyroid extract. *J Clin Invest* 38:1214–1221, 1959
5. Levi M, Peterson L, Berl T: Mechanism of concentrating defect in hypercalcemia. Role of polydipsia and prostaglandins. *Kidney Int* 23:489–497, 1983
6. Manitius A, Levitin H, Beck D, Epstein FH: On the mechanism of impairment of renal concentrating ability in hypercalcemia. *J Clin Invest* 39:693–697, 1960
7. Eigler JOC, Salassa RM, Bahn RC, Owen CA Jr: Renal distribution of sodium in potassium–depleted and vitamin D-intoxicated rats. *Am J Physiol* 202:1115–1120, 1962
8. Galla J, Booker B, Luke RG: Role of the loop segment in the urinary concentrating defect of hypercalcemia. *Kidney Int* 29:977–982, 1986
9. Peterson MJ, Edelman IS: Calcium inhibition of the action of vasopressin on the urinary bladder of the toad. *J Clin Invest* 43:583–593, 1964
10. Burch RM, Halushka PV: ^{45}Ca fluxes in isolated toad blatter epithelial cells: Effects of agents which alter water or sodium transport. *J Pharmacol Exp Ther* 224:108–117, 1983
11. Goldfarb S: Effects of calcium on ADH action in the cortical collecting tubule perfused in vitro. *Am J Physiol* 243:F481–F486, 1982
12. Marumo F, Edelman I: Effects of Ca^{++} and prostaglandin E_1 on vasopressin activation of renal adenyl cyclase. *J Clin Invest* 50:1613–1620, 1971
13. Beck N, Singh H, Reed SW, Murdaugh HV, Davis BB: Pathogenic role of cyclic AMP in the impairment of urinary concentrating ability in acute hypercalcemia. *J Clin Invest* 54: 1049–1055, 1974
14. Dousa TP: Cellular action of antidiuretic hormone. *Miner Electrol Metab* 5:144–158, 1981
15. Dousa TP: Renal action of vasopressin, in *Posterior Pituitary*, edited by Baylis PH, Padfield PC. New York, Dekker Inc., 1985
16. Kusano E, Murayama N, Werness J, Yusufi ANK, Christensen S, Homma S, Yusufi N, Dousa TP: Effect of calcium on the vasopressin–sensitive cAMP metabolism in medullary tubules. *Am J Physiol* 249:F956–F966, 1985
17. Takaichi K, Uchida S, Kurokawa K: High Ca^{2+} inhibits AVP dependent cAMP production in thick ascending limbs of Henle. *Am J Physiol* 250:770–776, 1986
18. Appelbloom JW, Brodsky WA, Tuttle WS, Diamond I: The freezing point depression of mammalian tissues after sudden heating in boiling water. *J Gen Physiol* 41:1153–1169, 1958
19. Kim JK, Summer SN, Berl T: The cyclic AMP system in the inner medullary collecting duct of the potassium–depleted rat. *Kidney Int* 26:384–391, 1984

20. EDWARDS RM, JACKSON BA, DOUSA TP: ADH-sensitive cAMP system in papillary collecting duct: Effect of osmolality and PGE_2. *Am J Physiol* 240:F311–F318, 1980
21. KIM JK, JACKSON BA, EDWARDS RM, DOUSA TP: Effect of potassium depletion on the vasopressin–sensitive cAMP system in rat outer medullary tubules. *J Lab Clin Med* 99:29–38, 1982
22. MOREL F, CHABARDES D, IMBERT–TEBOUL M: Methodology for enzymatic studies of isolated tubular segments: Adenylate cyclase, in *Methods in Pharmacology*, edited by MARTINEZ–MALDONADO M. New York, Plenum Press, 1976, pp. 297–323
23. SALOMON Y: Adenylate cyclase assay, in *Advances in Cyclic Nucleotide Research*, edited by BROOKER G, GREENGARD P, ROBISON GA. New York, Raven Press, 1979, pp. 35–55
24. LOWRY OH, ROSENBROUGH NJ, FARR AL, RANDALL RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265–275, 1951
25. IMBERT–TEBOUL M, CHABARDES D, MONTEGUT M, CLIGUE A, MOREL F: Impaired response to vasopressin of adenylate cyclase of the thick ascending limb of Henle's loop in Brattleboro rats with diabetes insipidus. *Renal Physiol* 1:3–10, 1978
26. JACKSON BA, EDWARDS RM, VALTIN H, DOUSA TP: Cellular action of vasopressin in medullary tubules of mice with hereditary nephrogenic diabetes insipidus. *J Clin Invest* 66:110–122, 1980
27. HALL DA, VARNEY DM: Effect of vasopressin on electrical potential difference and chloride transport in mouse medullary thick ascending limb of Henle's loop. *J Clin Invest* 66:792–798, 1980
28. DE ROUFFIGNAC C, ELALOUF JM, REINEL N, BAILLY C, AMIEL C: Similarities of the effects of antidiuretic hormone, parathyroid hormone, calcitonin and glucagon on rat kidney, in *Proc Int Cong Nephrol*, edited by ROBINSON RR. New York, Springer–Verlag, 1984, pp. 340–357
29. TEITELBAUM T, BERL T: Control of VP-stimulated cAMP synthesis in cultured rat inner medullary collecting tubule (RIMCT) cells: Role of calcium. *J Clin Invest* 77:1574–1583, 1986
30. MARX SJ, ATTIE MF, STOCK JL, SPIEGEL AM, LEVINE AM: Maximal urine concentrating ability. Familial hypocalciuric hypercalcemia versus typical primary hyperparathyroidism. *J Clin Endocrinol Metab* 52:736–740, 1981
31. HRUSKA KA, GOLIKORSKY M, SCOBLE J, TSUTSUM M, WESTBROOK S, MOSKOWITZ D: Effect of parathyroid hormone on cytosolic calcium in renal proximal tubular primary culture. *Am J Physiol* 251:F183–F198, 1986
32. SABATINI S: Parathyroid hormone inhibits water flow in the isolated toad bladder. *Am J Physiol* 250:532–538, 1986
33. RUGGLES BT, MURAYAMA N, WERNESS JL, GAPSTEEN SM, BENTLEY MD, DOUSA TP: The vasopressin sensitive adenylate cyclase in collecting tubule and a thick ascending limb of Henle's loop of human and canine kidney. *J Clin Endocrinol Metab* 60:914–921, 1985
34. MOREL F: Sites of hormone action in the mammalian nephron. *Am J Physiol* 240:F159, 1981